Assessments of molecular and biochemical analysis of the chemotherapeutic drugs adriamycin, cisplatin and fluorouracil on serum of male Albino rat

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ABSTRACT

Objective: The aim of the present investigation was to evaluate the cytotoxic effect of commonly used neoplastic drugs including adriamycin, cisplatin and 5-FU. Methods: Twenty-four mature male albino rats weighing approximately 130-150 g body weight were arranged into four groups; control, adriamycin (0.2 mg/kg.b.wt), cisplatin (0.2 mg/kg.b.wt.) and fluorouracil (20 mg/kg.b.wt.). The applied dose was intraperitoneally injected every other day for 21 days. Isoenzyme electrophoresis of alkaline-acid phosphatase, lactic dehydrogenase and glucose 6-phosphate dehydrogenase were investigated using electrophoresis. As well as, plasma content of vascular endothelial growth factor (VEGF), heat shock protein 70 (HSP-70), troponin-t, 8-hydroxy-2′-deoxyguanosine (8-OHdG), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) was measured by ELISA. Results: Different chemotherapeutic treatments exhibited alterations of the activity of isoenzymes including acid phosphatase, lactic dehydrogenase and glucose 6-phosphate dehydrogenase. As well as a significant reduction in serum contents of VEGF, HSP-70, bICAM-1 and VCAM-1, in contrary, an increase in troponin-t and 8-OHdG were observed. The present findings concluded that chemotherapeutic treatment interfere with cell function causing alterations in isoenzymes and reduction of cell growth by decreasing of VEGF, HSP-70 and cell adhesion molecules. As well as predict of cell damage as detected by increase of serum troponin-t and 8-OHdG.

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INTRODUCTION

Chemotherapeutic agents are used extensively in solid and hematologic malignancies and are increasingly used for their immunosuppressive properties in the management of inflammatory disorders. Chemotherapy involves the use of chemical agents to arrest the growth and eliminate cancer cells even at distant sites from the origin of primary tumor. However, it does not distinguish between a cancer and normal cells, and eliminates not only the fast-growing cancer cells but also other fast-growing cells in the body, including hair and blood cells. In fact, more than half of all people diagnosed with cancer receive chemotherapy regimen, that usually include drugs to treat cancer as well as drugs to help support the completion of the cancer treatment at the full dose on schedule. Chemotherapy is the standard therapy for advanced or metastatic disease and anti-tumor activity of drugs depends on the mechanism of action of how these agents are effective in killing the tumor cells or in preventing the growth of tumor cells. Single agent plays critical role in the local and regional control of malignant tumors. However, its efficacy can be limited by a number of factors including increased toxicity normal tissue injury, drug resistance and increased side effects. It is very apt notion that several potential mechanisms of resistance to anti-angiogenic drugs have been proposed and two main types of resistance can be distinguished: resistance of the tumor vasculature to the inhibition of VEGF and PDGF signaling (vascular resistance); and resistance of cancer cells to the hypoxic and nutrient-depleted microenvironment induced by antiangiogenic effects (hypoxia resistance - resistance to the effector mechanism of anti-angiogenic treatment). Chemotherapy with 5-fluorouracil is now indicated for adjuvant therapy of breast cancer (Kailajärvi et al., 2004). 5-fluorouracil has been used in the treatment of breast cancer, head and neck cancer, and gastrointestinal cancers. 5-Fluorouracil (5-FU) is currently being used as an anticancer drug to achieve optimal response and prolong the postoperative survival in patients of cervical cancer (Morris et al., 1999; Thomas et al., 2001). When given intravenously, it is metabolized in tissues to its active form, 5-fluoro-deoxyuridine-monophosphate, which inhibits thymidylate synthase. The drug is also catabolized primarily in the liver, as dihydouracil, and the reduced compound is then cleaved to α-fluoro-β-alanine, ammonia, urea, and carbon dioxide. Both the toxicity and anti-tumor effect are potentiated if the catabolism is blocked by inhibiting goal dihydouracil dehydrogenase.
Currently, cisplatin, doxorubicin and 5-FU have been extensively used for chemotherapy of various cancers, including that of the liver. However, while they generate acceptable outcome in chemotherapy of some cancers, they also exhibit severe toxicity and undesirable side effects. Extensive investigations have been conducted on the hepatotoxicity as well as general organ toxicity of these three anticancer drugs.

The aim of the present investigation was to evaluate the cytotoxic effect of commonly used neoplastic drugs including Adriamycin, cisplatin and 5-FU on the isoenzymes electrophoresis of alkaline-acid phosphatases, lactic dehydrogenases, glucose 6-phosphate dehydrogenase and some biochemical markers.

Materials and methods
Experimental Design:

Twenty-four mature male albino rats weighing approximately 130-150 g body weight were obtained from Hellwan Breeding Farm, Ministry of Health and were housed under controlled light conditions (12:12 hours light: dark) in the animal housing center of Mansoura University, Faculty of science, Egypt. Animals were maintained in an air conditioned and were provided with food and water ad-libitum. Standard diet composed of 50% grinding barely, 0.16% sodium deoxylcholate, 20 Mm Hepes/ NaOH, pH 7.0, and an appropriate amount of the enzyme was incubated at 37°C for 20min. The reaction was terminated by adding 0.5ml of 10% (w/v) trichloroacetic acid, followed by centrifugation at 2000 g for 10min. To 0.5ml of the supernatant, 3.0ml of chloroform/methanol/HCl (500:500:3, by vol.) and 0.8ml of IM-HCl were added. The sample was centrifuged at 2000g to release ALPase from plasma membranes by treatment with butan-l-ol, sodium deoxycholate. About 0.75 mg of the stored tissues was thawed, homogenized in 0.5 ml cold bi-distilled water and centrifuged (3000 rpm, for 5 minutes at 4°C). An amount of 50 µl of protein dye (1% bromophenol blue) and 20 µl of 2% sucrose is mixed. 30 µl of the mixture per gel slot were used to be applied for each sample in enzyme electrophoresis. Polyacrylamide gel electrophoretic (PAGE) profiles of LDH isoenzymes were utilized and software analysis of gel scans was performed. Electrophoresis was essentially carried out according to the protocol outlined previously . LDH isoenzyme bands were stained and visualized in the presence of L-lactate as the substrate according to the procedure of Shaw and Prasad . Then the stained gels were fixed in 7% acetic acid (v/v) and documented via scanning on HP Deskjet F370 All-in-One computer assembly.

Alkaline phosphatase (Al-Pase) electrophoresis:
Alkaline phosphatase activity was determined by using p-nitrophenyl phosphate substrate as described previously . A reaction mixture in 0.5ml containing 2mM-phosphatidylinositol, 0.16% sodium deoxycholate, 20 Mm Hepes/ NaOH, pH 7.0, and an appropriate amount of the enzyme was incubated at 37°C for 20min. The reaction was terminated by adding 0.5ml of 10% (w/v) trichloroacetic acid, followed by centrifugation at 2000 g for 10min. To 0.5ml of the supernatant, 3.0ml of chloroform/methanol/HCl (500:500:3, by vol.) and 0.8ml of IM-HCl were added. The sample was centrifuged at 2000g to release ALPase from plasma membranes by treatment with butanol-ol, sodium deoxycholate. About 0.75 mg of the stored tissues was thawed, homogenized in 0.5 ml cold bi-distilled water and centrifuged (3000 rpm, for 5 minutes at 4 C). An amount of 50 µl of protein dye (1% bromophenol blue) and 20 µl of 2% sucrose is mixed. 30 µl of the mixture per gel slot were used to be applied for each sample in enzyme electrophoresis. Then the stained gels were fixed in 7% acetic acid (v/v) and documented via scanning on HP Deskjet F370 All-in-One computer assembly.

Acid phosphatase electrophoresis
The enzyme determined according to Michelson and Dubois (1981). The procedure as previously mentioned before but with buffer system for the electrode, composed of tris – Borate (PH 8.9), incubating buffer at 5 °C for 20 minutes in 0.1M sodium acetate (pH 5.0). Staining was carried out using buffer containing 30 mg sodium alpha naphthyl phosphate, 0.25 ml 0.1 m MgCl2, 0.23 ml Mn d2 (10%), 5 ml NaCl (20%), 30 mg fast blue.

Lactate dehydrogenase isoenzyme electrophoresis
Lactate dehydrogenase (LDH: 3.1.1.27) is a tetrameric enzyme participates in carbohydrate metabolism by catalyzing the oxidation of lactate and reduction of pyruvate. The heart were separated at 13, 15, and 17 & 19 d prenatal and immediately stored at -80°C for enzymes electrophoresis. About 0.75 mg of the stored heart tissues was thawed, homogenized in 0.5 ml cold bi-distilled water and centrifuges (3000 rpm, for 5 minutes at 4°C). An amount of 50 µl of protein dye (1% bromophenol blue) and 20 µl of 2% sucrose is mixed. 30 µl of the mixture per gel slot were used to be applied for each sample in enzyme electrophoresis. Polyacrylamide gel electrophoretic (PAGE) profiles of LDH isoenzymes were utilized and software analysis of gel scans was performed. Electrophoresis was essentially carried out according to the protocol outlined previously. LDH isoenzyme bands were stained and visualized in the presence of L-lactate as the substrate according to the procedure of Shaw and Prasad. Then the stained gels were fixed in 7% acetic acid (v/v) and documented via scanning on HP Deskjet F370 All-in-One computer as-sembly.

Glucose 6-phosphate dehydrogenase isoenzyme:
The isoenzyme electrophoresis was determined according to Shaw and Prasad, 1970. The buffer system of the electrode is Tris- EDTA-Borate (TEB)(0.5M) ph 8.0. Staining buffer is composed of 30 mg NADP (betanicotinamide adenine dinucleotide phosphate), 20mg NTB9 nitroblue tetrazolium chloride), 2 mg PMS (phenazine methosulphate), 25 mg. Tris-Hcl (pH 7.1), 200 mg Na-glucose-6-phosphate and 50 ml distilled water.

Biochemical assessments of VEGF, HSP-70, troponin-t, ICAM-1, VCAM-1 and 8-OhdG:
The serum contents of VEGF were determined by using ELISA kit of the KO MA BIOTECH INC. However, HSP-70, troponin-t, ICAM-1, VCAM-1 were assayed by sing ELISA kit of the UsCN, Life science inc. The 8-OhdG is a competitive in vitro enzyme-linked immunosorbent assay (ELISA) for quantitative detection of the oxidative DNA adduct 8-OhdG. The assayed carried out using the kit of japan institute for the control of aging (JaICA) (Catalog. No. KOG-2005/E).

Results
Isoenzymes electrophoresis:
Four enzymes were assessed in serum of control and chemotherapeutic-treated rats including acid-alkaline phosphatases, lactic dehydrogenase and glucose 6-phosphatase
dehydrogenase. From table (1), the serum acid phosphatase isoenzyme express six fractions of isoenzymes in serum of rats subjected to flurouracil-treatment comparing to five in control. Their rate of diffusion and intensity were markedly increased. However in rats subjected to either adriamycin or cisplatin-treatment, the number of expressed bands were similar to control and the rate of diffusions were moderately affected (Fig.1A). In case of alkaline phosphatase, flurouracil –treatment exhibited similar number of bands as in control, but the rate of diffusion and intensities were highly changed. In case of adriamycin and cisplatin-treatment, extra band was developed and the rate of diffusions and intensities were altered (Fig.1B). From table (2), the serum lactic dehydrogenase isoenzyme express four fractions of isoenzymes as in control but their rate of diffusion and intensities were decreased. However in adriamycin and cisplatin-treatment, expression of extra band were detected but of decreased rate of diffusion and intensities (Fig.2A). In case of glucose 6-phosphate dehydrogenase, adriamycin –treatment exhibited similar number of bands as in control, but the rate of diffusion and intensities were decreased. In case of flurouracil and cisplatin-treatment, extra band was developed, but the rate of diffusions and intensities were depleted (Fig.2B).

**Serum biochemical markers:**

The applied chemotherapeutic drugs including flurouracil, adriamycin and cisplatin-treatment revealed marked depletion of vascular endothelial growth factor (VEGF) (Fig. 3B), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Fig. 3A). However, troponin-t, heat shock protein 70 (HSP-70) (Fig. 3B), and 8-hydroxy-2’-deoxyguanosine (8-OHdG) were apparently increase (Fig. 3A).

**Table (1). Serum acid (AcPase) & alkaline (AlPase) phosphatases isoenzyme electrophoresis of flurouracil (F), adriamycin (A), cisplatin (Cs)-treated male rats.**

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<tr>
<th>AcPase</th>
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<td>C</td>
<td>F</td>
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<td>Band</td>
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<tr>
<td>1</td>
<td>0.29</td>
<td>25.2</td>
<td>0.32</td>
<td>55.7</td>
<td>0.32</td>
<td>19.9</td>
<td>0.31</td>
<td>16.8</td>
<td>0.11</td>
<td>36.06</td>
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<tr>
<td>2</td>
<td>0.49</td>
<td>14.6</td>
<td>0.49</td>
<td>9.18</td>
<td>0.54</td>
<td>19.3</td>
<td>0.54</td>
<td>16.5</td>
<td>0.29</td>
<td>51.74</td>
</tr>
<tr>
<td>3</td>
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<td>26.5</td>
<td>0.57</td>
<td>8.72</td>
<td>0.65</td>
<td>34.4</td>
<td>0.64</td>
<td>29.7</td>
<td>0.88</td>
<td>12.18</td>
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<tr>
<td>4</td>
<td>0.76</td>
<td>14.7</td>
<td>0.69</td>
<td>10.3</td>
<td>0.86</td>
<td>12.5</td>
<td>0.84</td>
<td>11.6</td>
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<tr>
<td>5</td>
<td>0.85</td>
<td>19.1</td>
<td>0.77</td>
<td>4.18</td>
<td>0.95</td>
<td>13.9</td>
<td>0.93</td>
<td>25.4</td>
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<tr>
<td>6</td>
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<td>0.91</td>
<td>11.8</td>
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**Table (2). Serum lactic dehydrogenase (LDH) & glucose-6-phosphate dehydrogenase (G-6-PDH) isoenzyme electrophoresis of flurouracil (F), adriamycin (A), cisplatin (Cs)-treated male rats.**

|        | C     | F     | A     | Cs    |       |       |       |       |       |       |       |       |       |       |       |       |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Band   | RF    | %     | RF    | %     | RF    | %     | RF    | %     | RF    | %     | RF    | %     | RF    | %     | RF    | %     |
| 1      | 0.03  | 10.4  | 0.03  | 14.1  | 0.04  | 18.2  | 0.053 | 14.6  | 0.34  | 30.6  | 0.09  | 26.8  | 0.29  | 25.2  | 0.32  | 55.7  |
| 2      | 0.42  | 19.3  | 0.26  | 9.18  | 0.15  | 1.44  | 0.18  | 11.9  | 0.53  | 16.6  | 0.32  | 36.8  | 0.49  | 14.6  | 0.49  | 9.18  |
| 3      | 0.59  | 12.1  | 0.58  | 34.4  | 0.39  | 16.2  | 0.38  | 25.4  | 0.64  | 22.1  | 0.51  | 12.5  | 0.58  | 26.5  | 0.57  | 8.72  |
| 4      | 0.89  | 58.1  | 0.89  | 42.4  | 0.59  | 31.7  | 0.6   | 29.6  | 0.81  | 16.1  | 0.61  | 17.7  | 0.76  | 14.7  | 0.69  | 10.3  |
| 5      | 0.83  | 32.5  | 0.89  | 8.5   | 0.89  | 14.5  | 0.75  | 3.36  | 0.85  | 19.1  | 0.77  | 4.18  |       |       |       |       |
| 6      |       | 0.81  | 2.85  | 0.91  | 11.8  |       |       |       |       |       |       |       |       |       |       |       |
From the present findings, the applied anticancer drugs exhibited alterations of the serum isoenzyme electrophoresis of alkaline-acid phosphatases, lactate dehydrogenases and 6-phosphate dehydrogenases. The observed reduction of rate of diffusion and altered intensity of bands which express the activity of assayed enzymes represent the cytotoxicity of the assayed drugs in different body organs. Alkaline and acid phosphatase, have special biological functions involved in cellular transport, proliferation and differentiation. Measurement of the alpha 1 (fast liver) fraction of alkaline phosphatases in the serum for 217 cancer patients, 92 patients with nonmalignant hepatic affections and 131 controls, revealed that the alpha 1 fraction offers better global value (94%), sensitivity (96%), and specificity (93%) than gamma GT or total alkaline phosphatase determinations for the detection of liver metastases during cancer. Serum total alkaline phosphatase level was significantly elevated in patients and in patients with prostatic carcinoma with bone metastasis and bone metastasis. On the other hand serum LDH levels and electrophoresis of its isoenzymes may be of value in patients with ovarian dysgerminomas. Mercer (1990) reported that the prostatic acid phosphatase, placental alkaline phosphatase and lactate dehydrogenase isoenzymes were described as potential clinical usefulness to support a diagnosis of cancer and/or to assist in the monitoring of therapy. Gavriliuk et al. (2010) reported glucose-6-phosphate dehydrogenase in the blood and of patients with breast tumors with varying activity of the pathological process. The present findings revealed that the applied chemotherapeutic drug-treatment caused marked increase of troponin-t and 8-OHdG which are parallel with DNA damage and VEGF,HSP-70 and ICAM-1 and VCAM-1 which express the dramatic effects of organs dysfunction as a result of the cytotoxicity of the chemotherapeutic drugs. These may be attributed to the active DNA damage and cell loss. It is generally which were accepted for flurouracil, adriamycin, cisplatin, binding of cis-DDP to genomic DNA (gDNA) in the cell nucleus is the main event responsible for its interaction or properties which may inhibit transcription, and/or DNA replication mechanisms. Subsequently, these alterations in DNA processing would trigger cytotoxic processes that lead to cancer cell death.
Chemotherapeutic drugs represent one of the main targets of treatment cancer; however cytotoxicities in different body organs were reported. The most cytotoxic agents tested were methotrexate, mitoxantrone, adriamycin, mitomycin C and cisplatin. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) is the most popular marker for oxidative DNA damage, and has been reported to be elevated in patients with various malignancies including malignant lymphoma, T cell leukemia/lymphoma (ATL), acute leukemia, and myelodysplastic syndrome (MDS). Also, Akay et al. (2003) detected increased level of 8-OHdG levels in DNA from leukocytes of bladder cancer patients. Many research works had been reported that tumor EC have a suppressed expression of adhesion molecules, such as intercellular adhesion molecule-1/2 (ICAM-1/2) and vascular endothelial cell adhesion molecule-1 (VCAM-1), due to exposure to angiogenic factors such as vascular EC growth factors (VEGFs) and fibroblast growth factors (FGFs). In this context, VEGF-A is key mediators of these respective processes degradation of the extracellular matrix and angiogenesis resulted from tumour invasion and metastasis in human and canine neoplasia. Heat shock protein 70 was found to express during the multistep cholangiocarcinogenesis process (Sato et al., 2012). Cardiac cytotoxicity was assessed by increase of serum troponin. Serum concentrations of the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were markedly increased in pre-clinical or early cancer, and colorectal cancer patients.

The present findings revealed that the applied chemotherapeutic drug-treatment caused marked increase of troponin-t and 8-OHdG which are parallel with DNA damage and VEGF HSP-70 and prominent alteration in ICAM-1 and VCAM-1 which express the dramatic effects of organs dysfunction as a result of the cytotoxicity of the chemotherapeutic drugs

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